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Fish Immunotoxicology: Research at the Crossroads of Immunology, Ecology and Toxicology

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Abstract—The current testing paradigm used in ecotoxicological hazard and risk assessment is well appropriate for chemicals with non-specific modes of action, the question, however is, whether it is appropriate for specifically acting compounds as well. A specific mode of action that is shown by numerous environmental chemicals is immunotoxicity. Immunity is an ecologically relevant trait, which is of key importance for organism survival and population growth against the pressure of pathogens in their environment. However, the environment also imprints genotypic and phenotypic properties of the immune system. Immunologically relevant environmental factors include pathogens as well as toxic chemicals. A complicating factor in detecting immunotoxic effects is the fact that they may be not evident in the resting immune system, but only after immune activation by pathogen challenge. Consequently, risk assessment of chemical-induced disruption of immune function must focus not alone on the relationship between chemical exposure and the response of selected immune parameters, but it has to consider the complex functional properties of this system in its ecological context.

Keywords: environmental risk assessment, fish, immunotoxicology, ecological immunology

INTRODUCTION

Ecotoxicological hazard assessment currently relies on a rather small number of standardized laboratory tests using species from different trophic levels (autotrophs, primary and secondary consumers). These standard tests measure apical toxicological endpoints such as lethality, and give emphasis to acute effects of high exposure concentrations. Limitations of this primarily descriptive approach are evident: on the one hand, apical endpoints provide little insight into the underlying toxic processes and mechanisms, which complicates grouping of chemicals on the basis of common modes of action, as well as effect extrapolation across species (Eggen *et al*., 2004; Breitholtz *et al*., 2006; Segner, 2011); on the other hand, there is an ongoing debate if apical endpoints inform on the ecological consequences of toxic effects, since propagation from individual-level effects to

Table 1. Examples of toxicant effects on immune parameters and disease susceptibility of teleost fishes *in vivo*.

Chemical agent	Fish species	Immune effect	Reference
Metals			
Cd, Hg	*Oncorhynchus mykiss*	Reduced respiratory burst activity of phagocytes	Sanchez-Dardon *et al*., 1999
Organometals			
Organotin	*Oncorhynchus mykiss*	Reduced respiratory burst activity of leukocytes, enhanced pathogen susceptibility	Nakayama *et al*., 2009
Persistent organic pollutants, POP			
Aroclor 1248	*Ameiurus nebulosus*	Impaired IgM production, enhanced pathogen susceptibility	Iwanowicz *et al*., 2009
Polyaromatic hydrocarbons, PAH			
Benzo(a)pyrene	*Oryzias latipes*	Impaired lymphocyte proliferation, enhanced pathogen susceptibility	Carlson *et al*., 2002; 2004
Pharmaceuticals			
Diclofenac	*Salmo trutta*	Inflammatory responses	Hoeger *et al*., 2005
Pesticides			
Lindane	*Oncorhynchus mykiss*	Reduced oxidative burst, altered B cell function	Dunier *et al*., 1994
Esfenvalerate	*Oncorhynchus tshawytscha*	Enhanced pathogen susceptibility	Clifford *et al*., 2005
Endocrine disruptors			
17beta-estradiol	*Dicentrachus labrax*	Decreased plasma lysozyme and antibody titre	Thilagam *et al*., 2009
Nonylphenol	*Ictalurus punctatus*	Altered phagocyte activity	Rice *et al*., 1998
Mixtures			
Heavy oil	*Paralichthys oliveaceus*	Downregulation of virus defense-related genes; enhanced pathogen susceptibility	Nakayama, K. *et al*., 2008; Song *et al*., 2011

population-level effects is not linear but varies due to other factors including phenotypic plasticity, life history strategy, resilience/elasticity processes, or individual variations within populations (Kooijman, 1998; Rose, 2000; Calow and Forbes, 2003; Relyea and Hoverman, 2006; Segner, 2007).

Despite these *caveats*, the current ecotoxicological testing paradigm in use appears to work well to assess toxic hazards arising from high concentrations, short-term exposures, and non-specifically acting chemicals. The question, however, is whether it still works well for low concentrations, long-term exposures and specifically acting compounds, or whether such scenarios require the additional consideration of sub-organism responses and traits. One example to test this case is provided by endocrine disrupting compounds such as 4-nonylphenol. Standard ecotoxicological tests have shown that on acute exposure, this chemical induces lethality in fish in the mg/L range, and on this basis it has been categorized as a chemical with polar narcotic mode of action. However, at low concentrations (μg/L range), the relevant toxicity of 4-nonylphenol arises from interference with endocrine pathways what can result in impaired reproductive fitness. This effect quality of 4-nonylphenol had been missed by conventional ecotoxicological hazard assessment.

The objective of this communication is to discuss the challenges we face in ecotoxicological hazard assessment when it comes to specifically acting chemicals. This question will be discussed on the example of immunotoxic effects of chemicals upon fish. There is a steadily increasing number of publications, both from laboratory and field studies (see examples in Table 1), reporting on immunotoxic effects of environmental chemicals in fish (Zeeman and Brindley, 1981; Bols *et al.*, 2001; Rice, 2001; Burnett, 2005). Many "classical" environmental pollutants such as polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs) (Reynaud and Deschaux, 2006) are known to possess immunotoxic activities, but also emerging micropollutants, particularly endocrine disruptors and pharmaceuticals, seem to be able to modulate immune parameters of fish (Hoeger *et al.*, 2005; Casanova-Nakayama *et al.*, 2011). In the following, we will initially give a very short introduction to the fish immune system, then discuss the importance of immune defense in an ecological context, and finally address the impact of chemicals on immune system functioning.

The immune system of teleost fishes

The immune system is critical for survival and fitness of organisms in that it enables to distinguish between self, non-self (e.g., pathogens) and altered self. General design principles of immune systems include (i) combination of general and specific responses, (ii) division of tasks among specific immune cell populations, both resident and migratory ones, (iii) intensive communication and signaling among the various immune system components, (iv) a balancing of forces, e.g., between pro- and anti-inflammatory signals, and (v) extensive variability and continuous innovation to be able to cope with antigenic diversity, for instance, by polymorphism and polygeny (Trowsdale and Parham, 2004). In addition, the immune system must be in a state of preparedness even in the absence of any antigenic challenge, it must be in strategic locations within the organism in order to sense and communicate information on invading foreign material, and it must be able to rapidly replenish immune cells.

The immune system of fishes can be subdivided into broadly three categories which differ in the speed and specificity of response (Rice, 2001; Burnett, 2005). The first line of defense is presented by the external barriers separating the fish from its environment, i.e., the epithelia of skin, gills and alimentary canal. These epithelia work as mechanical barriers to invading pathogens, but they also contain chemical (antibodies, lysozyme, etc.) and cellular (immune cells) defenses. Inside the fish, the second immune category is formed by the innate immune system which enables a rapid response to invading pathogens. This system provides non-specific responses which are activated by pathogen associated molecular patterns (PAMP) that are common to many pathogens, for instance, bacterial lipopolysaccharide (LPS). The counterpart to PAMP on the pathogen side are pattern recognition receptors (PRR) on the host side which recognize either the foreign molecules or endogenous, host-derived alarm molecules (Magnadóttir, 2006). Main effector elements of the innate immune system of fishes include humoral factors such as lysozyme or complement factors, as well as phagocytic cells such as granulocytes, monocytes/macrophages and natural killer cells. The main functions of the phagocytic cells are to phagocytose tissue debris and microorganisms, to secrete immune response regulating factors and to bridge innate and adaptive immune responses. The third line of immune defense is the adaptive or acquired immune system, a set of humoral and cellular components that enable a pathogen-specific response. Adaptive immunity provides organisms with a mechanism for deriving an almost limitless variation from very few genes (Litman *et al.*, 2010), which represents a major advantage in the fight against genetically variable pathogens. Cells involved in the specific immune system are T- and B-lymphocytes which mediate the cellular and humoral responses, respectively. The lymphocytes possess antigen-specific receptors that are activated by antigenic peptides bound to Major Histocompatibility Complex (MHC) proteins that are displayed by either infected host cells (MHC Class I) or by professional antigen-presenting cells (MHC Class II). Although the characterization of piscine T-lymphocytes is by far not as progressed as in mammals, it is clear that fish possess both antigen-presenting T-helper cells (CD4-like) and cytotoxic T-cells (CD8-like) (Fischer *et al.*, 2006). Fish B-lymphocytes produce immunoglobulins which are primarily tetrameric IgMs (Warr, 1983), instead of the pentameric immunoglobulins of mammals.

The immune system of fishes is often considered to be a primitive one. This notion may be related to two observations: First, while higher vertebrates have two separate compartments to generate myeloid and lymphoid immune cell types (lymphoid: lymph nodes, thymus, spleen; myeloid: bone marrow), fish do not possess bone marrow or lymph nodes, and produce lymphoid and myeloid cells in the same compartments. Second, the adaptive immune of fish usually shows a rather slow response to infective pathogens, taking weeks instead of days as in mammals. In this context, it is important to remember that it is the group of

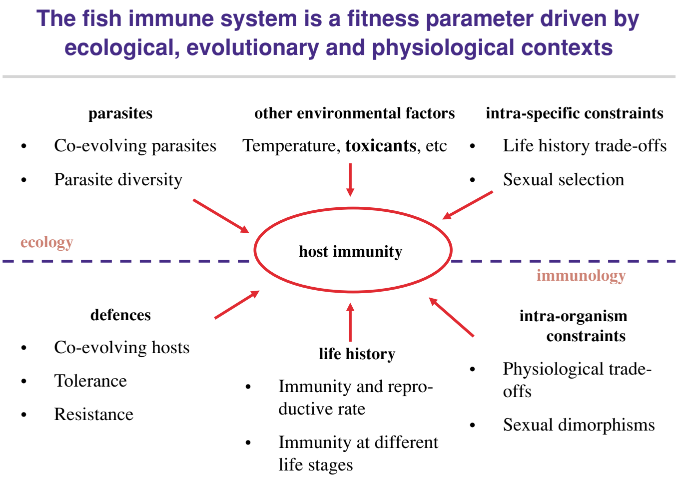

Fig. 1. Immune system structure, function and competence of an organism is shaped by the organism's evolutionary, ecological and life history contexts.

actinopterygian fishes which phylogenetically is the first vertebrate group to possess an adaptive immune system (Litman *et al.*, 2010). Despite these "primitive" criteria, the fish immune system is efficient enough to support ecological success of fishes in a wide range of environments and against a plethora of infectious pathogens.

Ecological immunology: the immune system in an ecological context

Immune system structure and function must not be seen in isolation, but has to be understood in the ecological context of the organism. The interaction between ecological and immunological properties and processes is the subject of the research field of "ecological immunology" which aims to understand host immunity in the broader framework of an organism's evolution, ecology and life history (Sheldon and Verhulst, 1996; Schulenburg *et al.*, 2009) (Fig. 1). There are three relevant aspects when considering relations between ecology and immunology: First, immunocompetence in the sense of an organism's ability to respond to a foreign antigen so as to minimize the fitness costs of infection (Owens and Wilson, 1999) is an important determinant of an organism's ecological fitness (Lazzaro and Little, 2009). Immunocompetence is closely related with fitness parameters such as survival, growth, breeding performance or fecundity (Lochmiller and Deerenberg, 2000). Achieving optimal immunocompetence is a key selective factor in reproduction, as sexual dimorphisms used for mate selection, e.g., ornaments, are considered to be proxies for good immunocompetence (cf. Nunn *et al.*, 2009). Accordingly, alterations of immunocompetence caused by genetic or physiological factors may translate into altered organism survival and reproduction. Second, ecological factors, both abiotic and biotic ones, shape an organism's immune system, thereby modulating its immunocompetence. Third—and this is a logical consequence of the two previous features—, the immune system is not a rigid, invariable entity, but is highly flexible in order modulate its activity in concert with current biotic and abiotic environmental conditions and the endogenous physiological status.

Ecological inputs on immune parameters of organisms can be manifold. Variable environmental conditions (and "environment" in this context means, e.g., temperature, availability of nutrient resources, genetic diversity of pathogens) favor variable immune traits as this will increase the chance to survive and reproduce in this environment. For instance, ambient temperature has been shown to modulate intensity as well as nature of fish immune responses to pathogens (Koellner and Kotterba, 2002; Xu *et al.*, 2011). Pathogen pressure is another ecological input that imprints host immunity, both over evolutionary and ecological time (Horrocks *et al.*, 2011). Pathogens are important regulators of their host populations (Hudson *et al.*, 1998; Webster *et al.*, 2011). The diversity of the parasite community influences the variation of the fish immune response, in particular of the MHC system (Scharsack *et al.*, 2007; Eizaguirre and Lenz, 2010). In fact, parasite and host immune diversity show rapid co-evolution, that is pathogen genetic diversity drives selection on the host immune system, and these changes in turn place selective pressure on the pathogens (Lazzaro and Little, 2009). The possible ecological relevance of this "race of arms" between pathogen and host is highlighted by "red queen hypothesis" which postulates that sexual reproduction has evolved as an adaptive strategy to genetically outrun rapidly co-evolving pathogens (Hamilton, 1980). Interestingly, there is evidence that sexual traits, which are used in sexual selection, mirror the immune status of their bearers; thus, immunocompetence is a key criterion in mate choice (Moller *et al.*, 1999, Faivre *et al.*, 2003).

The discussion on co-evolution already indicates that the ecological outcome of the confrontation of the host organisms with a pathogen depends both on endogenous factors and on environmental factors. Resistance/susceptibility is indeed a complex issue involving genetic and physiological factors, and often, it is the result of genotype by environment interactions (Lazzaro and Little, 2009). The diversity of defense options is important for the survival of organisms or populations in a variable world. At the same time, the immune system response has to be integrated in the context of other life history requirements. Activating the immune system is energetically costly (Ots *et al.*, 2001; Martin *et al.*, 2002), which may lead to resource competition and trade-offs with other energy-consuming life history functions (French *et al.*, 2009; Barber *et al.*, 2011). Key among these competing processes is the maintenance of health and the production of the offspring. Indeed, it has been shown that reproductive activities greatly influence an organisms's immune capacity. Likewise, investing in costly immunological defenses can impair reproductive function (Nordling *et al.*, 1998; French *et al.*, 2009).

The discussion above aimed to exemplify that immunity is a trait of organisms that clearly has an ecological dimension: the ecological context shapes genotypic and phenotypic properties of host immunity, and, vice versa, ecological processes are influenced by the immune status and capacity of the host. An interesting question is how this interactive system responds if toxicants come into play.

Toxicological immunology: the immune system in a toxicological context

Given the importance of the immune system for fitness and ecology of organisms, it is evident that a possible disrupting impact of environmental chemicals on the immune system may have far-reaching consequences. In fact, a wide variety of chemicals has been reported to impact immune parameters of teleost fishes (Zeeman and Brindley, 1981; Dunier and Siwicki, 1993; Anderson and Zeeman, 1995; Luebke *et al.*, 1997; Zelikoff *et al.*, 2000; Bols *et al.*, 2001; Rice, 2001; Burnett, 2005; Carlson and Zelikoff, 2008). A few examples from laboratory studies are presented in Table 1 in order to illustrate the diversity of immune-active chemicals and the diversity of immunological effects. Also in field studies, altered immune function of fish from polluted sites has been reported in a number of publications, for instance, immune-suppressed fish were observed at PAH-contaminated sites such as Chesapeake Bay (Luebke *et al.*, 1997) and Puget Sound (Arkoosh *et al.*, 1998). The pollution-induced immunotoxicity, alone or in combination with other stressors, can lead to enhanced disease susceptibility of exposed fish, which then may translate into population-level effects (Jacobson *et al.*, 2003; Spromberg and Meador, 2005; Loge *et al.*, 2005).

As the piscine immune cells and organs are closely associated with the blood system, and partly act as filtering system for the circulatory system, they are highly accessible to toxicants. Additionally, the immune system may be indirectly affected by toxicants via the neuro-endocrine system (Rice, 2001; Burnett, 2005, Casanova-Nakayama *et al.*, 2011). Thus, the question is not so much if toxicants do affect the immune system of fish, but the critical questions are (i) how to assess immunotoxic effects?, (ii) what are the mechanisms leading to immunotoxicity?, and (iii) what are the implications of the effects on immunocompetence and organism fitness?

The assays traditionally used to assess toxicant-induced perturbations of the immune system in fish fall mainly into two broad categories. One category includes assays that monitor immune structural and functional parameters, for instance, expression of immune mediators such as cytokines, phagocytosis assays, measurement of oxidative burst or of immune cell proliferation, changes of serum antibody titers, or immunopathological responses. The advantage of these assays and test parameters is that they show an effect of chemical exposure upon immune functional parameters in a straightforward way, a disadvantage, however, is that they do not necessarily inform if these effects translate into altered immunocompetence of the fish. The second category are pathogen challenge experiments, in which control and toxicant-treated fishes are exposed to an infectious pathogen in order to learn whether the chemical exposure increases pathogen susceptibility. An example how these assays can be used in combination are the studies of Carlson *et al.* (2002, 2004) to characterize benzo(a)pyrene immunotoxicity in medaka. Methodological approaches that have been found valuable in mammalian immunotoxicology such as immunpathology or assessment of changes in the composition of the immune cell populations are rarely used in studies with fish. Studies on chemical impact on the immune system of fish usually have reported immunosuppressive effects, although we know from studies with mammals that also immunostimulating effects, or autoimmune and allergic responses can occur—but these effect qualities have been rarely if ever studied in fish (Rice, 2001; Burnett, 2005).

The mechanisms through which environmental chemicals lead to changes of immune parameters of fish are little understood. Studies over recent years provided evidence that immune cells express receptors such as aryl hydrocarbon receptor (Nakayama, A. *et al.*, 2008) and estrogen receptors (Casanova-Nakayama *et al.*, 2011). Activation of these receptors by environmental chemicals such as PAHs or xenoestrogens may lead to downstream changes in immune gene expression (Haarmann-Stemman *et al.*, 2009; Jin *et al.*, 2010). If activation of these receptors can also lead to alterations in immune cell differentiation and recruitment, as it is discussed in mammals, remains to be shown for fish. Another mediator of immunotoxicity of environmental chemicals may be modulation of intracellular calcium levels, as shown by Betoulle *et al.* (2000) and Reynaud *et al.* (2004). The fact that immunotoxic mechanisms to date have been little studied in fish might be related in part to difficulties in identifying and separating individual immune cell types (due to the lack of markers and appropriate cell separation techniques). Fortunately, this situation is rapidly improving.

Do toxicant-induced alterations of molecular or cellular immune parameters implicate a compromised immunocompetence and enhanced disease susceptibility of the fish? This question is of paramount importance if we want to use immunotoxicity assays in environmental risk assessment. Here, one problem is that at the current state of knowledge on the fish immune system, it is difficult to interpret the relevance of a molecular or cellular change for the overall immunocompetence of the fish. In some cases, the interpretation appears to be rather straightforward, for instance, Iwanowicz *et al.* (2009) found that Aroclor 1248-exposed catfish displayed reduced serum bactericidal activity and antibody titers, and this suppression of anti-bacterial capacity translated into enhanced susceptibility to bacterial infection. However, given the complexity and network character of the immune system, associations between suborganism immune changes and organisms disease resistance are not always straightforward. A second problem in assessing the implications of suborganism immune changes for organism fitness is that the immunotoxic effects often may not be detectable in the resting immune system, but only in the activated immune system, when the fish is challenged with a pathogen (Koellner *et al.*, 2002). This may be illustrated by the study of Wenger *et al.* (2011) on the impact of exogenous estrogens on immunocompetence of rainbow trout: Juvenile rainbow trout were exposed for 4 weeks to concentrations of 17beta-estradiol which were sufficiently high to induce an estrogenic response, as evidenced from the induction of the estrogen biomarker, vitellogenin. Concurrent effects on the immune system—as assessed via the complement system—were not detected. At this stage of the investigation, the conclusion would have been that estrogenic exposure remains without effect on the immune status of trout. However, when the fishes were challenged with the bacterial pathogen, *Yersinia ruckeri*, then a significant difference between the control and the estrogen-exposed groups became evident: The estrogen-treated fishes, in contrast to the control fishes, were not able to up-regulate the expression of key complement genes in order to defend against the infectious pathogens. In line with this, the estrogen groups suffered significantly higher mortalities than the control groups. Apparently, the estrogenic treatment had an impact on immunocompetence of the trout. However, this impact was visible not in the resting but only in the activated immune system.

Conclusion: Implications for eoctoxicological risk assessment

The discussion above aimed on the one hand to point to the importance of the immune system for organism fitness and population growth. In their environment, organisms are constantly exposed to a wide and diverse range of pathogens, and an appropriate functioning of the immune system is the key success parameter in the "race of arms" between pathogens and hosts. On the other hand, the intention was to highlight that the immune system is regulated in a multifactorial way. This means that assessment of the hazard of immunotoxic chemicals must not only focus on the relationship between chemical exposure and the immune system, but it needs to take into consideration the complex functional properties and the ecological context of the immune system. It is also important to note that we deal with an entire system, not with a singular molecular or cellular endpoint—which means that we have to interpret the toxic effects in the physiological context of the organism. To date, ecotoxicological hazard assessment has given much emphasis to the physicochemical and structural properties of the toxicants as drivers of toxicity, however, for specific and complex effect qualities such as immunotoxicity, we have to give more emphasis on the structural, functional and ecological properties of the receptor system in order to get a clue on the possible adverse consequences of toxicant exposure.

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